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Large-Scale Analysis of Hepatitis C Virus Serological Typing Assay: Effectiveness and Limits

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The HCV (hepatitis C virus) Serotyping 1–6 Assay™ (Murex Laboratories) was evaluated on 303 French HCV-infected patients. Serological typing results were compared to the genotypes obtained from sequence analyses of the 5′ noncoding regions of the virus genome from 46 HCV-infected patients, and assay specificity was found to be high (97.6%). The serological typing assay, run in 257 consecutive HCV-infected patients, yielded an assay sensitivity lower (70.6%) than that previously reported. This finding was attributed mainly to nonreactive sera from human immunodeficiency virus (HIV)-positive patients ($P < 0.001$) and perhaps reflected cryoglobulin positivity in others. No anti-type 6 reactivity was detected, and the overall serological type distribution values for types 1 to 5 were 67.3, 7.9, 16.4, 6.6, and 0.9%, respectively. A higher prevalence of type 4 was noted among HIV-infected patients ($P < 0.001$). In addition, serotype 2 was significantly more frequent in cryoglobulinemia positive than in cryoglobulinemia-negative patients ($P < 0.05$). Although an initial high level (7%) of mixed serological typing reactivities was found, after predilution of serum only two mixed infections could be confirmed (0.9%). It is suggested, therefore, that mixed reactivities have to be interpreted carefully and retested with prediluted serum, particularly when the optical density of the reactivity is >2.5 or remains >0.4 after competition with all type-specific peptides. The high specificity and relatively good sensitivity even in immunocompromised patients obtained with this assay indicate that it can be used routinely. Because response to treatment is linked to HCV type, this assay could be used to identify HCV serotype to guide therapeutic decisions. ***J. Med. Virol. 55:18–23, 1998.***

KEY WORDS: HCV; HIV; serotyping assay; cryoglobulinemia; sequence analysis; diagnosis

INTRODUCTION

Hepatitis C virus (HCV) is the major cause of acute and chronic non-A, non-B hepatitis. HCV genomes have been classified into at least six major genotypes and numerous subtypes. This classification is based on extensive sequence comparison of either complete HCV genome or HCV core, envelope 1 (E1) gene [Bukh et al., 1993], nonstructural 5 (NS5) [Simmonds et al., 1994], 5′-noncoding (5′NC) [Simmonds et al., 1993a], and NS2 [Shukla et al., 1995] regions. This classification has obvious epidemiological interest, and there is now evidence of its clinical implications. The influence of HCV type on the development of cirrhosis has been reported [Pozzato et al., 1994; Féray et al., 1995; Pontisso et al., 1995; Silini et al., 1995]. However, it was observed recently that cirrhosis may occur irrespective of HCV type [Simmonds et al., 1996]. A relationship between HCV type and response to interferon therapy has been reported [Kanai et al., 1992; Yoshioka et al., 1992; Tsubota et al., 1993; Chemello et al., 1994]. Indeed, patients infected with HCV type 2 or 3 are more likely to have a sustained response to interferon-alpha (IFN-α) than patients infected with type 1, even after allowing for age and presence of cirrhosis [Simmonds et al., 1996].

For the determination of HCV genotype, sequence analysis, primer-specific amplification, probe-specific

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hybridization [Stuyver et al., 1993; Ravaggi et al., 1994], and restriction fragment length polymorphism (RFLP) analysis [Davidson et al., 1995] are expensive PCR-based procedures which are difficult to use on a routine basis. An alternative serological approach to identify the HCV-infecting type was developed recently [Simmonds et al., 1993b; Bhattacherjee et al., 1995]. This method is based on the detection of specific antibodies raised against the different HCV types. The "first-generation" serological diagnostic assay used an NS4-derived recombinant protein from HCV genotype 1a. In that assay, serological reactivity was less frequent when patients were infected with HCV genotype 2 or 3. Heterogeneity in the NS4 region was thought to account for this lack of reactivity, and epitope mapping of the NS4 protein has revealed the presence of different linear antigenic determinants among HCV genotypes [Simmonds et al., 1993b; Tsukiyama-Kohara et al., 1993]. Enzyme-linked immunosorbent assays (ELISA) based on peptides corresponding to the NS4 or the core regions have been used to characterize antibodies directed against HCV types 1 to 3 [Simmonds et al., 1993b; Tsukiyama-Kohara et al., 1993; Mondelli et al., 1994; Tanaka et al., 1994] and HCV types 1 to 6 (Bhattacherjee et al., 1995].

The HCV Serotyping 1–6 Assay™ (Murex Laboratories) was used recently to type anti-HCV antibodies from patients infected with HCV genotypes determined previously by RFLP or sequence analysis. Comparison between genotyping and serological typing with this assay showed a concordance of 72.6% to 97.3% and a sensitivity of 83.0% to 87.1% [Bhattacherjee et al., 1995; Van Doorn et al., 1996]. We evaluated this serological typing assay in 303 French HCV-infected patients. The high specificity of this method was confirmed on 46 samples genotyped previously by direct sequencing of the 5′NC region. This assay was then used on a routine basis in a larger HCV-infected population and in different subgroups, such as human immunodeficiency virus (HIV)-coinfected patients and patients with mixed cryoglobulinemia.

MATERIALS AND METHODS

Population

Forty-six patients were selected from a case-controlled study on mixed cryoglobulinemia conducted from 1991 to 1993 [Nguyen et al., 1998]. We also studied 257 consecutive chronically HCV-infected patients diagnosed between January and July 1996. All of these patients had HCV antibodies using two HCV-enzyme-linked immunosorbent assays (ELISA3; Ortho Diagnostics Systems, Raritan, NJ; Sanofi-Diagnostics Pasteur, Marnes-La-Coquette, France or Murex Diagnostics, Chatillon, France) and recombinant immunoblot assay (RIBA3; Ortho Diagnostic Systems). Among the 280 HCV-infected patients tested, 55 were positive for anti-HIV antibodies (Sanofi-Diagnostic Pasteur; Behring). Among the 67 individuals tested for the presence of circulating cryoglobulins, 24 had mixed cryoglobulinemia.

Cryoglobulinemia Detection

Blood samples were drawn into a prewarmed Vacutainer system and allowed to clot at 37°C for 30 to 60 min before centrifugation at 2,000 *g* for 10 min at 37°C. Sera were stored at 4°C for 1 week. After 3 washes with saline buffer, the cryoprecipitate was detected by means of an automated immunonephelometric assay (BNA, Behring) whose detection limits are 4 mg/l for IgG, 3.4 mg/l for IgA, and 3.1 mg/l for IgM.

HCV Genotyping

The HCV genotypes of the viruses infecting 46 patients were determined by sequencing of the 5′NC region. Viral RNA was obtained from 100 μl of serum by proteinase K digestion, phenol-chloroform/isoamyl alcohol extraction, and isopropanol precipitation. First-strand cDNA synthesis was carried out with 5 μl of RNA, using 10 pmoles of reverse primer CV320 (position −7 to −22: CGGTCTACGAGACCTC) and 100 IU of Superscript II reverse transcriptase (GIBCO-BRL, Gaithersburg, MD) in 25 μl of reaction mixture at 42°C for 45 min. Hot-start PCR was performed with 5 μl of cDNA, using primers of the 5′NC region (biotinylated sense primer CV49b position −291 to −272: GAGGAACTACTGTCTTCACG and antisense primer CV293, position −30 to −49: ACTCGCAAGCACCCTATCAG) and 1.5 U of Taq polymerase in 80 μl of reaction mixture (Perkin-Elmer Cetus, Norwalk, CT). Amplification was initiated with five cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by 35 cycles at 94°C for 1 min, 62°C for 45 sec, and 72°C for 45 sec. The specificity of the amplification was confirmed by Southern hybridization using a 5′[γ-^{32}P]ATP-labeled internal oligonucleotide SF1 (CGCATGGCGTTAGTATGAGT).

The polymerase chain reaction (PCR) product (5′NC region) was sequenced directly with 33 μl of the PCR mixture added to 25 μl of streptavidin-coated magnetic beads (Dynabeads™; Dynal, Fort Lee, NJ). After denaturation (5 min in 100 μl of 0.2 M NaOH, 0.2 mM ethylenediaminetetraacetic acid), the sense strand (with the biotinylated primer) was magnetized. The supernatant containing the antisense strand was neutralized with 20 μl of 2 M ammonium acetate, pH 5.3, and precipitated with 200 μl of ethanol. The magnetic bead pellet was washed first in 150 μl of 10 mM Tris (pH 7.5), 1 mM EDTA, and 2 M NaCl, then in 150 μl of 10 mM Tris (pH 7.5) and 1 mM EDTA. Sequences of both strands were determined using the dideoxy sequencing method, primer CV49 or CV293, and the Sequenase II™ sequencing kit (USB, Cleveland, OH), as described by the manufacturer. Sequences of both strands were read independently by two observers and aligned using CLUSTAL V software. Discrepancies were resolved by a third reading. Multiple sequence alignment of NS5 sequences from −244 to −69 was carried out with CLUSTAL W and the PILEUP module of the Genetic Computer Group (GCG) package. Prototype sequences of known genotypes were included to facilitate geno-

TABLE I. Comparison of Serological Typing and Genotyping Results*

	Serological typing										%	%
Genotyping	1	2	3	4	5	6	NT	NR	M	Total	Spec.	Sen.
1[a]	17						5	3		25	100	68.0
1a	5						1			6	100	83.3
1b	12						4	3		19	100	63.1
2		5					1			6	100	83.3
3			5				2	3	1	11	83.3	54.5
4				1			2			3	100	33.3
5										1	100	100
6										0		
Total	17	5	5	1	1	0	10	6	1	46	97.6	69.4

*NR, nonreactive; NT, non-type-specific; M, mixed; Spec., specificity, or number of correct serotypes/total number of serotypable samples; Sen., sensitivity, or number of serotypable samples/total number of samples.
[a]1 = 1a + 1b.

type assignment. These sequences were obtained from GenBank and corresponded to type 1a (accession number M62321, isolate HCV-1), 1b (M58335, HCV-BK), 2 (D00944, HC-J6), 3 (D17763, NZL1), 4 (M84862, Z6 and L29594, G22), and 5 (L29585, BE96).

HCV Serological Typing

HCV serological typing was carried out using the HCV Serotyping 1–6 Assay™. This assay uses synthetic peptides corresponding to various NS4 antigens from HCV types 1, 2, 3, 4, 5, and 6, coated onto a solid phase. To minimize cross-reactivities between epitopes common to different types, a competitive reaction was performed and patients' sera were coincubated with five of the six HCV-genotype peptides in free solution. A control-reaction assay with no competing peptide and a competition well with the six free peptides, referred to as "competing solution-all" by the manufacturer, were included for each sample. The manufacturer's criteria recommended for interpretation were applied. These results scored the patients' antibodies as either type-specific (1 to 6), mixed, non-type-specific, or nonreactive. Ten nontypeable sera and 15 sera suggesting mixed infections were tested under various predilution conditions. A 1/5 to 1/50 predilution of serum was used according to the optical density (OD) value measured in the "competition solution-all" well. Sera were prediluted 1/5, 1/10, 1/20, and 1/50 when the OD value of the "competition solution-all" well was, respectively, <1, $\geqslant 1$, <1.5, $\geqslant 1.5$, <2, and >2.

Statistical Analysis

Statistical analysis was undertaken using a χ^2 test with Yates's correction when the size of the calculated class was <5.

RESULTS

The specificity of the HCV Serotyping 1–6 Assay™ (97.6%) was calculated by comparing the results of serotyping to those of genotyping (by nucleotide analysis of the 5′NC region) for 46 HCV-infected patients (Table I). In one sample, mixed serological reactivities (1 and 3) were detected, although a single HCV genotype (type 3) was identified. The overall sensitivity of the assay was 70.6%, with 47 samples being nonreactive and 42 being non-type-specific (Table II). Among these 42 untypeable sera, 10 were retested after predilution: they remained non-type-specific or became nonreactive.

Anti-type I antibodies were predominant (Table II). In contrast to previous findings, type-specific sensitivity was lower in our population. In addition, anti-type 4 antibody was surprisingly high in a European population (Table II). In HIV-positive patients, the sensitivity of the serological typing assay was significantly lower than for the HIV-negative subgroup (Table III). HCV–HIV-coinfected patients had more nonreactive sera than HIV-negative patients ($P < 0.001$, Table III). We also noted that, among the typeable sera, type 4 was significantly more frequent in our HCV–HIV-coinfected population ($P < 0.001$), with type 4 prevalence being second among anti-HIV-positive patients (7/36, 19.4%).

Among the 24 patients with mixed cryoglobulinemia, the sensitivity of the assay was lower than for the overall population or for cryoglobulinemia-negative patients but not significantly so (Table III). By contrast, serotype 2 was significantly more frequent in cryoglobulin-positive patients than in their cryoglobulinemia-negative counterparts ($P < 0.05$). Eight of the patients were treated by hemodialysis. The sensitivity of the serotyping assay for these patients was low (62.5%), mainly because of non-type-specific reactivities, but the number of patients was too small for the results to be statistically significant. Could the immunodepression associated with chronic dialysis have an influence on these findings, as suggested recently (M. Bogart, personal communication)?

Fifteen samples (7%) were suggestive of mixed infections. Most of these (n = 10) generated high OD (>2.5) in the reaction well. Furthermore, after competition with the "competing solution-all," the OD remained >0.4 for 9/15 (60%) of these sera. By contrast, for typeable sera, the OD after competition with the "competing solution-all" remained >0.4 in only 39/199 (19.6%) of the sera ($P < 0.01$). After predilution of the sera exhibiting mixed reactivities, nine became monotype-specific, while three showed non-type-specific reactivity, one became nonreactive, and the last two continued to express mixed reactivities, both directed against types 1 and 3.

TABLE II. Sensitivity of the Serological Typing Assay Among the Total Population and Distribution of the 214 Type-Specific Serological Reactivities

Serotype	n	% Frequency among typable sera	% Sensitivity
Type-specific sera	214		70.6%
1	144	67.3	
2	17	7.9	
3	35	16.4	
4	14	6.6	
5	2	0.9	
6	0	0.0	
Mixed	2	0.9	
Nonreactive sera	47		15.5%
Non-type-specific sera	42		13.9%
Total	303		100%

DISCUSSION

The aim of the study was to evaluate the ELISA-based (Murex HCV Serotyping 1–6 Assay™) for specific antibody responses to HCV genotypes 1 to 6. This evaluation entailed two steps. The results of the serological typing assay were first compared with those of the genotype as determined by sequence analysis. Among 46 HCV-infected patients, the specificity of the assay (97.6%) was confirmed to be high [Bhattacherjee et al., 1995; Van Doorn et al., 1996]. This specificity permitted use of this assay routinely. Therefore, the second step of the study was to evaluate this serological typing assay in a larger population of 257 HCV-infected patients and in different subgroups.

The distribution of HCV types obtained by serological testing was similar to that described previously using primer type-specific amplification or Inno-LiPA™ (Innogenetics, Zwijndrecht, Belgium) among French HCV-infected patients from Paris and its suburbs [Pol et al., 1994; Nousbaum et al., 1995; Pawlotsky et al., 1995b]. Although type 1 was the most prevalent, serological typing did not differentiate between types 1a and 1b. Whether or not this distinction is important to predict the prognosis of liver infection is still under investigation. Type 3 has been shown to be common among intravenous drug users (IVDUs) [Pawlotsky et al., 1995b]. A high percentage (6.6%) of HCV type 4, which is common in the Middle East and in Northern and Central Africa [Chamberlain et al., 1997], was observed. Its presence in the suburbs of Paris could reflect the close historical relationship France shares with African countries and immigration flow.

Serotype 4 was significantly more frequent (19.4%) in the HIV–HCV-co-infected patients than in the HIV-negative patients (4.3%) ($P < 0.001$). Type-4 HCV infection might reflect either imported infections from Africa or the spread of this type among IVDUs, as suggested recently [Gabastou et al., 1996]. This dissemination among French patients might be important because the prognosis of type 4 infection needs further investigation [El-Zayadi et al., 1996]. As new anti-HIV treatments are promising, HCV-type 4 infection might induce symptomatic liver damage before HIV-linked immunodepression develops.

In the mixed-cryoglobulinemia patients, serotype 2 was significantly more frequent ($P < 0.05$) than in the overall population or among cryoglobulinemia-negative patients. This finding differs from two other studies [Willems et al., 1994; Pawlotsky et al., 1995a], which did not find a specific HCV type in association with cryoglobulinemia. This discrepancy could be explained by the smaller size of the populations studied by those investigators. Indeed, Zignego et al. [1996] used two different genotyping assays and concluded that type 2 might be linked to cryoglobulinemia. Our serological findings also suggest a speicific link between HCV type 2 and cryoglobulinemia, which needs to be further investigated.

Why was the sensitivity of the HCV Serotyping 1–6 Assay™ in our population lower (70.6%) than reported by others (87.1% and 83.0%) [Bhattacherjee et al., 1995; Van Doorn et al., 1996]? The lower sensitivity of the assay might be linked to differences between the populations studied. Indeed, the sensitivity of the assay was found to be lower in HIV-positive than in HIV-negative patients, and this difference was linked to a significantly higher percentage of non-reactive sera ($P < 0.001$). This lack of reactivity could be explained by a lower amount of specific antibodies in these immunocompromised patients. Indeed, antibody specificity decreases with progression of immunodepression in HIV-positive patients [Lane et al., 1983]. The sensitivity of the assay was also lower in patients with mixed cryoglobulinemia than in the overall population and in cryoglobulinemia-negative patients, but these differences did not reach statistical significance due to the small number of patients tested. Could the presence of anti-NS4 antibodies in cryoprecipitates explain the decreased assay sensitivity in the cryoglobulinemia-positive population? We consider that the lower sensitivity obtained with the population investigated was associated with the inclusion of HIV-positive patients and, possibly, cryoglobulinemia-positive patients as the previous series did not include HIV-infected patients [Bhattacherjee et al., 1995; Van Doorn et al., 1996].

Finally, predilution of the sera helped to clarify the type of potential mixed infections detected by the serological typing assay. We initially detected 15 sera dem-

TABLE III. Comparison of Serotype Distribution and Serotyping Sensitivity Between HIV-Negative and HIV-Positive Patients (Cryoglobulinemia Patients Excluded) and Cryoglobulinemia-Positive and Cryoglobulinemia-Negative Patients

Serotypes (n = 177)	HIV-positive (n = 55)	HIV-negative (n = 225)	χ^2 test	Cryoglobulinemia-positive (n = 24)	Cryoglobulinemia-negative (n = 43)	χ^2 test
1	24(66.7%)	112(68.3%)	ns	8(53.3%)	22(64.7%)	ns
2	1(2.8%)	12(7.3%)	ns	4(26.7%)	3(8.8%)	$P < 0.05$
3	4(11.1%)	28(17.1%)	ns	3(20.0%)	7(20.6%)	ns
4	7(19.4%)	7(4.3%)	$P < 0.001$	0(0.0%)	2(5.9%)	ns
5	0(0.0%)	2 (1.2%)	ns	0(0.0%)	0(0.0%)	ns
6	0(0.0%)	0(0.0%)	ns	0(0.0%)	0(0.0%)	ns
Mixed	0(0.0%)	2(1.8%)	ns	0(0.0%)	0(0.0%)	ns
NR	13(23.6%)	30(13.3%)	$P < 0.001$	4(16.7%)	3(7.0%)	ns
NT	6(10.9%)	31(13.8%)	ns	5(20.8%)	6(14.0%)	ns
Sensitivity	65.5%	72.9%	$P < 0.001$	62.5%	79.1%	ns

NT, non-type-specific antibodies; NR, nonreactive; Sensitivity, number of serotypable samples/total number of samples; ns, nonsignificant difference.

onstrating mixed reactivities (7%), which was a surprisingly high level. Using genotyping methods, such a high number of mixed infections has been reported only in hemophiliacs [Jarvis et al., 1994], and no such patients were present in our population. However, a similar percentage (5.6%) was reported when the serological typing assay was applied to a broad population of European HCV-infected patients [Simmonds et al., 1996]. These mixed reactivities might reflect successive infections with different genotypes, as suggested by Simmonds et al. [1996]. However, we noted that these sera with mixed reactivities had very high OD and were poorly neutralized by soluble peptides. When these 15 sera were retested after predilution, only two mixed infections (2/214, 0.9%) could be confirmed. It is suggested, therefore, that the mixed serotypes as determined using the HCV Serotyping 1–6 Assay™ should be carefully controlled and retested with prediluted sera, particularly when the OD in the reaction control well is >2.5 or when the OD in the "competing solution-all" well remains >0.4. These mixed reactivities might reflect an excess of cross-reacting antibodies.

In conclusion, this highly specific assay seems to be useful. In HCV-infected, immunocompromised patients, the sensitivity was at least 65% but needs to be improved further. Because the response to IFN-α seems to be type-dependent, it is important that the HCV-infecting type be readily detectable so as to guide therapeutic decisions. This serotyping assay could be used for that purpose as it is rapid and can be automated. It is therefore less time-consuming and less expensive than genotyping methods. These latter methods might be applied when the serological typing assay is inconclusive, for epidemiological or taxonomic purposes, and to analyze viral resistance and pathogenesis. Therefore, both serological and molecular approaches are complementary ways to study HCV-type infections.

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